The potential of glycomics as prognostic biomarkers in liver disease and liver transplantation

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Abstract

The study of glycomics is a novel and fascinating approach for the development of biomarkers. It has become clear that in the field of liver disease specific glycomic patters are present in specific disease states, which has led to the development of diagnostic biomarkers. In this manuscript, we will describe two new applications of this technology for the development of prognostic biomarkers. The first biomarker is associated with the risk of hepatocellular carcinoma development in patients with compensated cirrhosis. The second biomarker is present in perfusate and is related to the risk of primary non function occurrence after liver transplantation. The technology used for these biomarkers could easily be implemented on routine capillary electrophoresis equipment. (Acta gastroenterol. belg., **2019**, 82, **309-313**).

Key words: glycomics, glycosylation, glycan, prognostic, biomarker, cirrhosis, hepatocellular carcinoma, primary non function

Glycomics : an introduction

One of the most important medical evolutions of the last decade is the development of personalized medicine. The success of personalized medicine depends on having accurate diagnostic tests that identify patients who can benefit from targeted treatment strategies (1). Over the last years, a large range of different *omics* have led to the development of valuable biomarkers, based on genomics, proteomics, transcriptomics, metabolomics etc. (2). Only few attention has been paid to the study of glycomics, an attractive though not well known technology that can lead to the development of biomarkers in a variety of diseases (3-5).

In human cells proteins are frequently modified with complex glycan structures. This process, called glycosylation, is the most frequent posttranslational modification (6) Three major types of glycosylation have been observed : *N*-linked glycosylation of asparaginases (6) *O*-linked glycosylation of serine and threonine (7) and glycosylphosphatidyl inositol derivatization of the carboxyl-terminal carboxyl groups (8). Here, we will focus on the *N*-glycosylation of human proteins.

N-glycosylation is essential for cell viability. It is strictly regulated by specific enzymes (9) and at least half of the genes functioning in this biosynthetic pathway have been conserved through evolution. These glycan structures are involved in diverse biological processes including protein folding and conformation, cell structure and stability and cell-matrix and cell-cell interaction (9).

Alterations in the abundance of particular N-glycans reflect an altered physiological state. This makes N-glycans particularly attractive for biomarker development. Furthermore, N-glycoproteins are highly regulated during growth and differentiation and alterations in protein N-glycosylation patterns correlate with developmental programs like morphogenesis, proliferation and apoptosis (10).

Over the last 10 years, it has become clear that serum proteins display typical glycoalterations in humans with chronic liver disease as opposed to healthy adults (3). This observation is supported by the fact that the majority of circulatory proteins are secreted by the liver and thus these glycoalterations can reflect pathological processes in the liver. A minority of serum glycans are attached to IgG and are secreted by B-cells (11,12), and are involved in inflammatory processes.

Standardized protocols for sugar labeling and its application on a DNA sequencer using DNA sequencer assisted fluorophore assisted capillary electrophoresis (DSA-FACE) has provided researchers with a technical environment that allows glycome analysis of the liver patient (13,14).

This pioneering work resulted more than 10 years ago in the development of the first glycomics-based biomarker for the diagnosis of fibrosis and cirrhosis, called the GlycoFibroTest (11) and the GlycoCirrhoTest (15,16). Other technologies including mass spectrometry, chromatography and lectin based assays have been applied for glycomic analysis but these cannot combine a high diagnostic accuracy with high throughput abilities at an affordable cost, as is needed for clinical implementation (13).

Glycomics for diagnosis of liver disease

Although non-invasive markers for the quantification of liver fibrosis have become mainstream in clinical practice during the last years (17), invasive liver biopsy was until then the standard of care for fibrosis

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and cirrhosis assessment (18). Making use of different technical approaches, several groups have shown consistent changes in the whole serum glycome in patients with chronic liver disease, according to increasing levels of liver fibrosis leading to the development of specific biomarkers for liver fibrosis (11,12,19,20) and cirrhosis (16). Glycomic profiling of serum of patients with hepatocellular carcinoma (HCC) has also lead to consisting observations. These findings include increased core fucosylation (21-24) and multibranching glycans (25; 26). The use of alpha-foeto-protein-L3 (AFP-L3), the fucosylated L3 fraction of AFP could dramatically increase the performance of this widely used biomarker (27).

In the field of non-alcoholic steatohepatitis (NASH) there is an ongoing need for biomarkers that can differentiate between simple steatosis and NASH (28). We have shown (29-31), amongst others (32-35), that the serum glycomic profiling can identify NASH patients among patients with simple steatosis.

Glycomics as prognostic biomarkers in liver disease

Over the last year, we have shifted our attention from the development of diagnostic biomarkers to prognostic biomarkers, based on glycomic profiling.

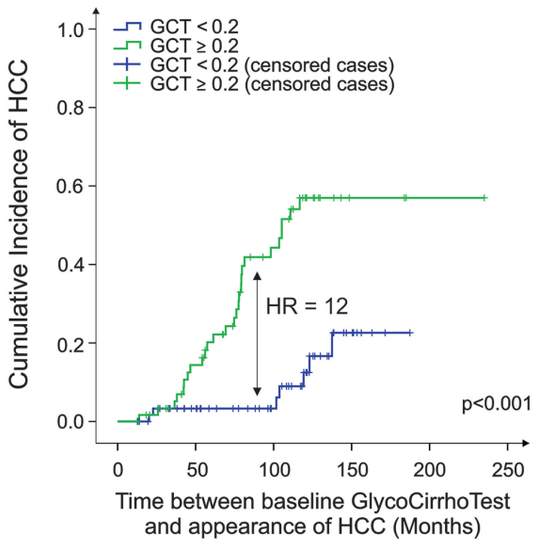

Figure 1. — Cumulative incidence curve representing the risk for developing hepatocellular carcinoma according to value of the GlycoCirrhoTest (adapted from Verhelst et al. (40)). The cohort was divided according to the GlycoCirrhoTest threshold and monitored for the appearance of hepatocellular carcinoma. Blue line, patients with a GlycoCirrhoTest value <0.2; green line, patients with a GlycoCirrhoTest above or equal to the threshold of 0.2. These patients show an increased risk for hepatocellular carcinoma development (HR ¼ 5.1; 95% CI, 2.2–11.7; P < 0.001). Censored cases (as indicated by crosses on the cumulative incidence curves) died, underwent liver transplantation, or were lost to follow-up.

Risk of HCC development in compensated cirrhosis

Liver cancer is the sixth most common cancer worldwide and the fourth cause of cancer-related death (36). Cirrhosis is the main risk factor for the development of HCC. Although European (37) and American (38) guidelines advocate ultrasonography-based screening for early HCC with or without measurement of AFP, adherence to these screening programs is disappointingly low (39) and the incidence of HCC is rising (36).

Recently, we showed that the GlycoCirrhoTest, a glycomics-based serum marker, could radically change this approach. While the current screening strategy is a one-size-fits-all approach, we showed that the use of the GlycoCirrhoTest was able to assess the risk of HCC development in patients with compensated cirrhosis and this could allow for a personalized screening protocol.

In a French cohort of 125 patients, a single measurement of the GlycoCirrhoTest, which is based on the measurement of 2 single glycans (a bisecting N-acetylglucosamine (GlcNAc)- containing N-glycan and a triantennary N-glycan) on serum glycoproteins was able to distinguish (Hazard ratio 12.1 ; 95% CI, 2.8–51.6 ; p< 0.01) between patients with a high and a low risk of HCC development during the following 7 years (40) (Figure 1). The median follow-up time in this study was 6.4 years. In a multivariate Cox Regression model including GlycoCirrhoTest, AFP and FIB-4 only GlycoCirrhoTest showed a significant and independent association with HCC development during follow up.

This finding is supported by a strong pathophysiological rationale. The enzyme N-acetyl glucosaminyl transferase III responsible for the formation of bisecting GlcNAc residues, which are the driving glycans of the GlycoCirrhoTest, is increasingly expressed in cirrhotic nodules (41,42). It is conceivable that with more hepatocytes actively dividing in such nodules, the risk for propagation of oncogenic mutations increases and hence the risk for HCC rises. Therefore a true marker for such nodular regeneration in liver cirrhosis should also be a good risk marker for HCC, as validated here for the GlycoCirrhoTest (16).

The findings of this study could be the basis for a radical change in the approach of the cirrhotic patient. In this era of personalized medicine such tools are highly valuable. Based on the result of the GlycoCirrhoTest, patients could be stratified in a low-risk or high-risk group for HCC development. These patients could be offered personalized screening regimens. For example, this study teaches us that patients with a low GlycoCirrhoTest value have an extremely low chance of HCC development (less than 5%) in the first 3 years of follow-up, whereas this risk is higher than 40% in the patients with a high GlycoCirrhoTest value. It could make sense to offer the GlycoCirrhoTest low patients a yearly screening examination, whereas the GlycoCirrhoTest High group should be followed every six months as it is performed now.

Yet, these results need to be interpreted with caution, as this was a monocentric study with a considerable but not extremely high number of patients included. However, from a statistical point of view the use of cross-validation and bootstrap validation confirmed the strong association of GlycoCirrhoTest with HCC development. This is a unique cohort with clinical data and serum that has been collected prospectively with a median follow-up time of almost 7 years. The search for similar cohorts that would allow validation has been difficult and disappointing. A prospective validation will take several years before useful results will be available.

The next step will be a pharmaco-economic evaluation of this strategy. If this strategy proves to be non-inferior to the current screening strategy, the financial repercussions could be very attractive. The reduction of visits by 50% in the GlycoCirrhoTest Low patients represents a real cost benefit, especially in countries where health insurance is not universal and where the distance to medical care can be larger than in Belgium.

In conclusion, the observation that GlycoCirrhoTest is a predictor of HCC risk in patients with compensated cirrhosis could be a highly-expected game-changer that answers the desire for personalized medicine and cost reduction in modern medicine.

Outcome after liver transplantation

For the first time glycomic profiling of perfusate was performed using DNA sequencer assisted fluorophore assisted capillary electrophoresis on a DNA sequencer as developed by the team of Callewaert et al. (14,16). From a theoretical point of view, perfusate analysis is an attractive alternative for liver biopsy or serum markers for the assessment of viability of the liver in liver transplantation. Perfusate is believed to represent the condition of the entire liver parenchyma and it is easy to collect in large volumes. However, only few perfusate markers have previously shown any value in the prediction of graft and patient outcome after liver transplantation (43). Our study confirms the potential of perfusate analysis to guide donor allocation. In a cohort of 66 liver transplant patients, three patients developed primary non function (PNF) and based on the glycomic signature of perfusate before liver transplantation we were able to predict the occurrence of PNF after liver transplantation with 100% accuracy (44). These findings were validated in an independent cohort (Figure 2).

A few reflections should be made in the appraisal of these results. First, although this multicentric study provides a strong proof-of-concept, a larger multicentric prospective validation is needed (and ongoing). Second, the glycome signature is based on an increase of undergalactosylated glycans. The increased abundance of undergalactosylated glycans is a phenomenon that we also encountered in NASH patients (30) and in the serum of liver transplant patients that are at greater risk of early graft loss (Verhelst et al, unpublished results). We know from previous

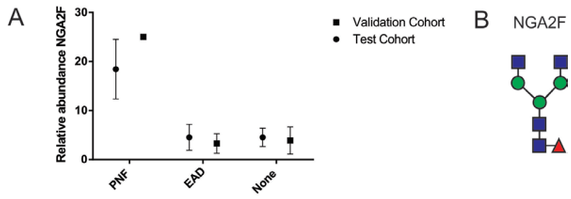

Figure 2. — Overview of the relative abundance of NGA2F, an undergalactosylated glycan in patients who developed primary non function (PNF) after liver transplantation. A. NGA2F shows 100% accuracy for prediction of PNF ($p<0.001$). p values were calculated using Mann Whitney U-test. B. Structure of NGA2F. The symbols used in the structural formulas are as follows: blue square, b-linked GlcNAc; red triangle indicates alpha,beta-1,3,6-linked fucose; green circle, a/b-linked mannose. PNF: primary non function. EAD: early allograft dysfunction. Adapted from Verhelst et al. (44).

reports that the undergalactosylated glycans in serum are present on IgG and not on liver-derived proteins (11,12). We measured IgG levels in perfusate and found their presence in the perfusate fluid (unpublished data). The levels of IgG were not increased in PNF patients compared to the non-PNF group. This implies that the undergalactosylation in this perfusate is a real glycomic alteration. The pathophysiological rationale is far from clear. However, it makes sense that the failing liver is a "stressed" organ, eg. by increased ischemia-reperfusion injury, with an important upregulation of inflammatory pathways, which is reflected by the increased abundance of undergalactosylated glycans. These livers are suffering and fail to develop the regeneration capacity to start functioning after liver transplantation.

Third, the accuracy for the prediction of PNF was extremely high (100%). On the other hand we found no clear relationship between the perfusate glycome profile and the occurrence of early allograft dysfunction (EAD). We speculate that this might be related to the fact that EAD is a complex and multifactorial syndrome that is not only related to the quality of the donor liver graft but also to recipient characteristics and intra- and postoperative events. This information will of course not be captured in the pretransplant glycome profile. On the other hand, PNF is mainly related to very low quality grafts (45), which can be captured quite convincingly by the glycome profile.

If we can confirm the predictive power of perfusate glycomics for the occurrence of PNF this could have an important impact on allocation practices. Organ allocation these days is more "art" than "science". Our tools for graft assessment are limited to a clinical appreciation and the use of scores like the Donor Risk Index (DRI) or Eurotransplant-DRI (46,47), which lack the prognostic power in individual patients. On the other hand, we are faced with a decreasing quality of organs. All strategies that aim at expanding the donor pool like the use of DCD donors or the use of elderly donors have an additional negative impact on donor quality (48).

A reliable biomarker that can predict organ failure with 100% certainty could help us to discard these unsafe organs from the donor pool and safely use donor organs where the clinician is in doubt regarding the quality. We remind the reader that our data were obtained in real clinical practice where all organs, including the PNF patients, were considered safe by the transplant team.

Although we are convinced of the real clinical value of this glycomic marker for PNF, the current approach as published in the study reveals a major obstacle towards implementation in clinical practice. The technique that we used in the publication has a turn-around time of 48 hours. In a concept where donor graft quality would be assessed before liver transplantation, this does not make any sense and makes this technique obsolete. New technical developments should decrease the turnover time to an acceptable 30 minutes (Callewaert et al., unpublished data).

Conclusion

In this work we explored the potential of glycomics to detect prognostic biomarkers in liver disease and liver transplantation. First we demonstrated that the GlycoCirrhoTest, a biomarker that was formerly developed for the diagnosis of cirrhosis, is not only a diagnostic marker of cirrhosis, but also a prognostic marker that can predict whether a patient with com-pensated cirrhosis has a low or a high risk to develop HCC. This is a new tool that could be used in the follow up of patients with cirrhosis, as these patients need six-monthly screening for HCC by ultrasonography. This GlycoCirrhoTest could help in the development of personalized screening protocols for cirrhotic patients according to the value of this test.

In the second section we assessed the potential value of glycomics to develop prognostic biomarkers in the field of liver transplantation. We showed that it was technically feasible to measure glycome profiles on perfusate, the fluid in which the liver is transported from the organ donor to the liver recipient. We successfully identified a specific glycome signature that is 100% predictive of a patient's risk for developing primary non-function, a dramatic complication in the first hours upon liver transplantation that requires urgent retransplantation. This biomarker could prevent the transplantation of unsafe donor livers.
In conclusion, the work described here adds to the increasing evidence that the whole serum protein glycome is a robust surrogate marker of processes that affect the liver. Furthermore, we were able to identify several potential prognostic biomarkers with real clinical utility in cirrhosis and liver transplantation. Validation studies of the most promising glycomics-based biomarkers are ongoing.

Conflict of interest

N. Callewaert is listed as a co-inventor on a patent on GlycoCirrhoTest that is owned by VIB vzw and has been licensed to Helena Biosciences. X. Verhelst and H. Van Vlierberghe are listed as a co-inventors on a patent on the perfusate marker for PNF as described in the manuscript that is owned by Ghent University.

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